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Interlaboratory Comparison of the ASTM (American Society for Testing and Materials) Bioconcentration Test Method Using the Eastern Oyster

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16. ABSTRACT

Four laboratories (one EPA and three contract labs) participated in a bioconcentration interlaboratory comparison study using the eastern oyster (Crassostrea virginica). Three chemicals were selected for study, including p,p'-DDE, pentachlorophenol (PCP), and 1,2,4-trichlorobenzene (TCB). The test method used was that of the American Society for Testing and Materials (ASTM) "Standard Practice for Conducting Bioconcentration tests with Fishes and Saltwater Bivalve Molluscs" (Draft 9). Calculated steady-state bioconcentration factors (BCFs) for p,p'-DDE ranged from 27,000 to 92,000; that for PCP was 34 to 82; and that for TCB was 115 to 264. The high-to-low ratio (an estimated of the "worse case" variability, derived by dividing the highest BCF by the lowest BCF for each chemical) was 3.4 for p,p'-DDE, 2.4 for PCP and 2.3 for TCB. These results indicate that the ASTM Method can generate reproducible results even though the participating laboratories varied in their experience and in their water quality characteristics and were widely separated geographically.

17. KEY WORDS AND DOCUMENT ANALYSIS

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Interlaboratory Comparison of the ASTM Bioconcentration Test Method Using the Eastern Oyster

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ABSTRACT: This paper summarizes the results of an interlaboratory comparison conducted by four laboratories for bioconcentration factor (BCF) testing using the eastern oyster (*Crassostrea virginica*) and the organic chemicals pentachlorophenol (PCP), 1,2,4-trichlorobenzene (TCB), and p, p′-DDE. The bioconcentration test method used was Draft 9 of ASTM Proposed Standard Practice for Conducting Bioconcentration Tests with Fishes and Saltwater Bivalve Molluscs. The mean steady state BCFs and high to low BCF ratios (*H/L* ratio) for PCP, TCB, and p,p′-DDE were 64 (2.4), 168 (2.3), and 52 600 (3.4), respectively. The mean high to low ratio for all three chemicals was 2.7, which represents the extreme estimate of variability. The mean *H/L* ratios for the time to 90% uptake were 6.2 for PCP and 5.8 for TCB while the ratio for time to 90% depuration for all three chemicals was 3.3. The results were reproducible, considering the varying degrees of experience between participants in conducting BCF tests, the wide geographic distribution between participants, and the different dilution water characteristics.

KEY WORDS: aquatic biology, bioconcentration, fishes, bioconcentration factor, log *p*, pentachlorophenol, 1,2,4-trichlorobenzene, p,p′-DDE, eastern oyster, *Crassostrea virginica*

The phenomenon of bioconcentration, the uptake of chemicals into an organism from water, is recognized by industry and regulatory agencies as a characteristic of some chemicals requiring early identification in the hazard assessment process. There are several examples of chemicals (for example, polychlorinated biphenyl [PCBs], dichlorodiphenyltrichloroethane [DDT], and so forth) that bioconcentrate in aquatic organisms [*1,2*] causing a loss in

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the usability of a fishery, or promote adverse reproductive effects on predators of the fishery [*3,4*].

It is therefore important that bioconcentration test methods used in the hazard assessment process for new chemicals be standardized and that the precision of such tests be determined. In 1977 the U.S. Environmental Protection Agency's (EPA) Office of Pesticides and Toxic Substances was required to publish guidelines for a variety of tests deemed important in the hazard assessment of chemicals. One of these tests was the bioconcentration of chemicals in either fish or shellfish. At this time, no interlaboratory comparison (round robin) had been conducted on a bioconcentration test method. The only generally accepted method available was that under development (Draft 9) by ASTM entitled Proposed Standard Practice For Conducting Bioconcentration Tests with Fishes and Saltwater Bivalve Molluscs [*5*]. The EPA received permission to use the draft method for the interlaboratory comparison.

This paper describes the results of twelve bioconcentration tests using the eastern oyster (*Crassostrea virginica*) as the test species. Four laboratories participated in the interlaboratory comparison of the chemicals pentachlorophenol (PCP), 1,2,4-trichlorobenzene (TCB), and 1,1-dichloro-2,2-bis (p-chlorophenyl) ethylene (p,p′-DDE).

Procedure

Test Animal

The test species selected for the bioconcentration study was the eastern oyster, *Crassostrea virginica*. This species is important economically as a human food source and ecologically in its wide distribution on the Atlantic and Gulf Coasts. *C. virginica* and *C. gigas* (a Pacific Ocean species) are particularly valuable in monitoring programs as pollution indicators because of their ability to bioconcentrate many organic chemicals [*6*]. Oysters used by all laboratories were either collected from relatively unpolluted estuarine sites (Laboratories 1, 2, and 3) or purchased from a commercial source (Laboratory 4). All oysters tested ranged from 2- to 6-cm umbo to distal valve edge height.

Test Chemicals

The test chemicals used in the interlaboratory comparison were pentachlorophenol (PCP), 1,2,4,-trichlorobenzene (TCB), and p,p′-DDE and were selected specifically to encompass a wide range of bioconcentration factors as predicted from their octanol/water partition coefficients [*7*]. In addition, the chemicals were selected because of their relative ease of chemical analysis and low mammalian toxicity.

Participating Laboratories

The four laboratories that participated in the study represented two major saltwater bodies of the United States: the Pacific Ocean (one laboratory) and the Gulf of Mexico (three laboratories). Each laboratory used natural, unfiltered seawater for their bioconcentration tests.

Performance Evaluation

A major source of variability in the interpretation of bioconcentration tests is derived from the chemical analysis of tissues and water for the respective chemicals. For this reason, we evaluated the analytical chemistry performance of all participating laboratories to identify any significant variability. Contaminated tissue and water samples were provided to each laboratory by messenger on the same day, and reports of the analysis were required no later than 30 days following delivery of the samples. Only one participant (Lab 4) failed to satisfy this deadline.

The lead laboratory, the Environmental Research Laboratory (ERL), Gulf Breeze, FL, conducted a large number of analyses for each chemical (in water and tissue) to establish relative mean concentrations, standard deviations, and corresponding 99% confidence intervals. The participants received the results of the ERL performance evaluation, and those deemed acceptable were allowed to proceed with testing. Participants displaying poor performance were cautioned about their respective methods. Improvements were suggested and changes were made to improve their performance.

Test Method

In addition to the performance evaluation package, each participant was provided information on the test chemicals to choose properly the test concentrations and sampling periods, as prescribed in the ASTM method [5]. According to the ASTM method, concentrations of the test chemical in water should not elicit adverse effects on the test organisms and, therefore, should not exceed 0.1 times the 96-h EC_{50} (effect was based on reduced shell deposition [8]). Consequently, participants were provided with the oyster EC_{50} values for each chemical (PCP, 104 μg/L; TCB, >500 μg/L; and DDE, 14 μg/L). For the bioconcentration tests, the lowest value allowed by ASTM [5] for the chemical in water is three times the analytical detection limit for each chemical tested. Therefore, the concentrations selected for bioconcentration testing were between the EC_{50} and these values.

The sampling intervals for each chemical are dictated by the logarithm of the octanol/water partition coefficient (log *P*); the higher the log *P*, the longer the time between samples and, therefore, the longer the exposure. We pro-

vided each laboratory with the estimated log P for each chemical (2.89 for PCP, 3.32 for TCB, and 5.69 for p,p′-DDE), as generated by Veith et al [7].

In addition, each laboratory was requested to provide the lead laboratory with control and experimental oysters (collected at steady state) from each test to determine lipid content. This procedure was suggested in the ASTM method [5] for lipophilic compounds.

The ASTM document specifies certain methods and conditions in order for a test to be considered satisfactory. These conditions are always associated with the word "must." For example, Section 10.9.3 states (in part) that a "group of organisms must not be used for a test if the individuals appear to be diseased or otherwise stressed or if more than 3% die during the 48 h immediately before the beginning of the test." Suggestions for good test practices are generally phrased in words such as "should," rather than "must." For example, Section 10.3.2 states (in part) that "in any single test, all molluscs should be from the same year class and the valve height of the largest mollusc should be no more than 1.5 that of the smallest mollusc." Each laboratory's final report was carefully scrutinized to determine if the "musts" in the ASTM document were fulfilled. If not, an attempt was made to determine if the test results were affected.

Chemical Methods

Each of the four laboratories used standard analytical techniques for the analysis of PCP, 1,2,4-TCB, and p,p′-DDE in seawater and in oyster tissues. No details are presented here. However, specific procedures of most laboratories were judged adequate during the performance evaluation; those judged inadequate modified their procedures before definitive testing.

For water analyses, 1 L of seawater was partitioned at least twice in a 2-L separatory funnel with the appropriate solvent for each chemical. After phase separation, the solvent phase was concentrated for injection into a gas chromatograph for quantitation.

For oyster tissue analyses, individual oysters (four from each sampling period) were weighed wet without fluids, homogenized, and extracted with the appropriate solvent at least three times. The extract was placed into a concentrator tube, concentrated to 1 mL in preparation for cleanup.

Concentrations of each chemical in water and oysters are quantitated by peak height area using gas chromatography.

Statistical Methods

The NONLIN [9] statistical model of Bahner and Oglesby was used to determine bioconcentration factors and to describe the uptake and depuration of the test chemicals in oysters.

Results

Pentachlorophenol, Trichlorobenzene, and DDE Bioconcentration Factors

The calculated steady state bioconcentration factors (BCFs) for the three chemicals tested by the four participating laboratories are listed in Table 1. Steady state BCFs were provided for each chemical by all laboratories.

Pentachlorophenol (PCP)—Results of the PCP bioconcentration studies indicated that the steady state bioconcentration factors (BCFs) varied from 34 to 1294. The obvious disparity of Lab 4's BCF (1294) with the other three prompted a request that Lab 4 verify its calculations. Lab 4 could not determine any errors. However, a review of the PCP data in their final report indicated that the measured PCP in water samples varied from 0.42 to 22.6 μg/L. This accounted for the extremely large standard deviation (3.75) about the arithmetic mean measured PCP value of 2.15 μg/L (Table 2). For these rea-

TABLE 1—*Calculated steady state bioconcentration factors (BCF) for pentachlorophenol (PCP), 1,2,4-trichlorobenzene (TCB), and p,p′-DDE, using the eastern oyster,* Crassostrea virginica.

Laboratory	PCP	TCB	p,p′-DDE
1	82	147	42 652
2	34	115	48 602
3	76	264	91 826
4	1 294[a]	148	27 191
	$\bar{x}$ = 64	168	52 568
	s = 26	65	27 684
	H/L ratio = 2.4[b]	2.3	3.4

[a]Data not considered in $\bar{x}$, s, and H/L ratio.
[b]H/L ratio = largest BCF ÷ smallest BCF.

TABLE 2—*Nominal and mean measured water concentrations* (μg/L) *of pentachlorophenol (PCP), 1,2,4-trichlorobenzene (TCB), and p,p′-DDE for each laboratory in bioconcentration interlaboratory comparison studies.*

	PCP		TCB		p,p′-DDE	
Laboratory	Nominal	Measured	Nominal	Measured	Nominal	Measured
1	5.0	3.7 ± 1.4	5.0	4.4 ± 0.54	1.0	0.38 ± 0.19
2	10.0	9.5 ± 1.2	25.0	8.8 ± 1.9	1.0	0.26 ± 0.1
3	10.0	11.3 ± 1.7	50.0	52.1 ± 3.1	1.2	0.66 ± 0.31
4	NR[a]	2.15 ± 3.75	NR	4.2 ± 0.3	NR	0.43 ± 0.07

[a]Not recorded in final report.

sons and because Lab 4's value was an obvious outlier, we deleted this test from further discussion in the interlaboratory comparison.

Labs 1, 2, and 3 provided PCP bioconcentration factors of 82, 34, and 76, giving a mean of 64 ± 26. The ratio of the highest BCF value divided by the lowest (the high to low ratio, *H/L* ratio) for the three laboratories was 2.4.

1,2,4-Trichlorobenzene (TCB)—The results of the TCB bioconcentration tests (Table 1) indicate that the mean BCF for the four laboratories was 168 ± 65; the *H/L* ratio was 2.3.

p,p′-DDE—The results of the p,p′-DDE bioconcentration tests (Table 1) show that the steady state BCFs ranged from 27 200 to 91 800, indicating that the mean BCF for this insecticide was 52 600 ± 27 700. The *H/L* ratio was 3.4.

Table 2 compares the measured water concentration to nominal (intended) concentration for each laboratory and each test chemical. Nominal concentrations were not provided by Lab 4 in the final report.

Uptake rates (the rate at which test chemicals accumulated in oyster tissues when oysters are held in seawater with the test chemical) were calculated using NONLIN [9] for all participating laboratories, except for Labs 2, 3, and 4 in tests with p,p′-DDE (Table 3). The average time for 90% uptake of PCP was 45 h, that for TCB was 68 h. These values correspond with their estimated log *P* (2.89 and 3.32); however, among the four laboratories there was substantial variability (*H/L* ratio was 6.2 for PCP and 5.8 for TCB). Data from Lab 4's PCP test were not considered because of the reasons presented earlier. Time to 90% uptake for p,p′-DDE exceeded the 28-day (672-h) depuration time for all but Lab 1, and therefore mean time for all four laboratories could not be calculated. NONLIN [9] calculated the time to 90% depuration for Lab 1 as 25 days (600 h).

TABLE 3—*Calculated time, in hours, to attain 90% of steady state and time to attain 90% depuration from steady state for pentachlorophenol (PCP), 1,2,4-trichlorobenzene (TCB), and p,p′-DDE, using the eastern oyster,* Crassostrea virginica.

	Time to 90% Uptake, h			Time to 90% Depuration, h		
Laboratory	PCP	TCB	p,p′-DDE	PCP	TCB	p,p′-DDE
1	34	91	600	8	24	582
2	88	23	>672	9	17	654
3	14	25	>672	17	30	960
4	>672[a]	134	>672	25	90	NSD[c]
	$\bar{x}$ = 45	68	...	15	40	732
	s = 38	54	...	8	34	200
	H/L ratio[b] = 6.2	5.8	...	3.1	5.3	1.6

[a] Not used in calculation of $\bar{x}$, s, or *H/L* ratio.
[b] The greatest time to 90% uptake or depuration divided by the shortest time.
[c] No significant depuration detected over 672 h in DDE-free seawater.

Depuration rates (the rate at which test chemicals were purged from oyster tissues when animals were held in seawater free of the test chemical) were calculated for all participating laboratories and test chemicals except for Lab 4 and p,p′-DDE (Table 3). The time required for 90% depuration of PCP from the respective steady state value ranged from 8 h (Lab 1) to 25 h (Lab 4), giving an H/L ratio of 3.1. For TCB, time to 90% depuration ranged from 17 h (Lab 2) to 90 h (Lab 4), giving an H/L of 5.3. For p,p′-DDE, depuration rates were calculated for only three laboratories (1, 2, and 3), giving an H/L ratio of 1.6. Lab 4 depuration data indicated that there was no significant loss of p,p′-DDE over the 28-day depuration period. Reasons for this phenomenon are not clear, but Lab 4 was consistently higher in the time required to depurate all three test chemicals.

Analysis of the Adherence of Laboratories to the ASTM Methods and Scope of Work

Final reports from each laboratory participating in the bioconcentration/interlaboratory calibration were carefully reviewed to determine if the "must" requirements of the ASTM document and the contract's Scope of Work were implemented. Tables 4, 5, and 6 address these requirements and their implementation.

TABLE 4—*Performance of each laboratory in adhering to diluent and test water condition requirements specified in ASTM bioconcentration test procedures. "Yes" designates compliance with the requirements; "No" designates that the required dilution water observations were not made, or that test water conditions were not within required limits.*

Laboratory	Salinity	pH	DO	Particulates	TOC or COD	Total Organo-chlorine COD	Temperature, 28°C
				DILUENT WATER QUALITY			
1	yes	yes	yes	yes	no	yes	...
2	yes	yes	yes	no	no	no	...
3	yes	yes	yes	yes	yes	no	...
4	NR[b]	NR	NR	NR	NR	NR	...
				TEST WATER CONDITIONS[c]			
1	yes	yes	yes	...	...	...	yes
2	yes	yes	yes	...	...	...	yes
3	No[d]	yes	yes	...	...	...	yes
4	NR	NR	NR	...	...	...	yes

[a]"Should" requirements in the ASTM procedure.
[b]NR = not reported in the final report.
[c]"Must" requirements in the ASTM procedure. Salinity = >12 g/kg, pH is < 0.8 units, and DO = >60% saturation.
[d]Acceptable salinity only for the pentachlorophenol test.

TABLE 5—*Performance of each laboratory in adhering to ASTM bioconcentration test procedures regarding eastern oyster* (Crassostrea virginica) *size, loading, acclimation time, and mortality during acclimation and testing. "Yes" indicates compliance with the requirements; "No" indicates noncompliance or data were not reported.*

Laboratory	Oyster Size, largest < 1.5 × smallest	Mortality		Acclimation Time, >4 days[a]	Loading, >1 L/oyster/h[a]
		Acclimation, <3%	Test, <10%		
1	yes	yes	yes	yes	yes
2	yes	yes	yes	yes	yes
3	yes	yes	NR	yes	yes
4	yes	yes	yes	yes	no[c]

[a]A "must" requirement in the ASTM procedure.
[b]NR = not reported with final report.
[c]Loading was acceptable only for the pentachlorophenol test.

TABLE 6—*Analyses of lipids (dry-weight) in oysters obtained from laboratories participating in the "round-robin" tests. Oysters were collected at steady state and analyzed individually.*

Laboratory	N[a]	Mean Percentage Lipids		
		Neutral	Polar	Total
1	5	2.4	...	...
2	8	3.7	4.1	7.8
3	8	6.0[b]	6.0	12.0[b]
4	8	3.6	5.2	8.8

[a]N = number of individual oysters measured to determine mean percentage lipid.
[b]Significantly ($\alpha = 0.5$) higher in percentage lipid than Labs 2 and 4, using ANOVA and Duncan's multiple range test.

Each laboratory, except Lab 4, provided sufficient information to make an adequate appraisal of diluent water quality and test water conditions used in the bioconcentration tests (Table 4). The only information provided by Lab 4 was the temperature of the test water. For diluent water quality, Labs 1, 2, and 3 each measured salinity, pH, and dissolved oxygen (DO), and those values were within prescribed limits. Particulates were measured by Labs 1 and 3, but not by Labs 2 and 4. Only Lab 3 measured total organic carbon (TOC). Total organochlorines were measured by Lab 1 only.

Measurement of test-water conditions varied among laboratories (Table 2). Salinity was within prescribed limits (> 12 g/kg) for Labs 1 and 2, but Lab 3 maintained salinity above 12 g/kg only during the PCP test. As stated earlier, Lab 4 reported only test temperatures in their final report. The report did state, however, that "open ocean seawater" was used for their test; from this, one could assume that salinity was >12 g/kg. The remaining test water conditions (namely, temperature, pH, and DO) were measured by Labs 1, 2, and 3 and were within the prescribed limits.

Assuming that total organochlorine pesticides in the test water (other than the test compounds) were sufficiently low that the oysters were not adversely affected, we would expect that only the low salinity data from Lab 3 might adversely affect the test results. Salinity in two of their tests dropped as low as 2 g/kg. Pumping rates of eastern oysters exposed to these low salinities were undoubtedly reduced; however, Lab 3 made no mention of reduced pumping rates (valve closure) or reduced deposition of feces or pseudofeces. The high steady state BCFs associated with Lab 3 tend to indicate that the oysters were unaffected.

Lipid Analyses at Steady State

Each contract laboratory was requested to provide the lead laboratory (Lab 1) with oysters collected at steady state from each test to determine the oyster lipid content. Oysters were received frozen and remained so until lipid analyses were conducted. Eight individual analyses were made on oysters from Laboratories 2, 3, and 4 to determine polar, neutral, and total percentage-lipid (dry weight). Results of these analyses are listed in Table 6. Lab 1 lipid analyses were incomplete because procedures used to determine lipid differed from those used for the other laboratories. Also, oysters from Lab 1 were used to identify potential toxicant-related effects on lipid content of which none was statistically different and, therefore, not used for comparison with other laboratories.

A comparison of the percent lipid values of Labs, 2, 3, and 4 (Table 6) with their respective steady state BCF (Table 1) demonstrates a potential relationship. Oysters from Lab 3 contained significantly higher neutral and total lipids than those of Labs 2 and 4; correspondingly, oysters of Lab 3 were also higher than those of the other two laboratories in the steady state BCFs for all three chemicals tested. We did not attempt to adjust the steady state BCFs for lipid content, because precise wet weight could not be determined for the oysters. However, recommendations for changes in the ASTM procedure to utilize fully lipid data for BCF normalization follow in the Discussion section of this report.

Discussion

The results of the bioconcentration interlaboratory tests indicate that the ASTM method can generate reproducible results. The mean H/L ratio for the calculated BCF for all three chemicals and four laboratories (excluding Lab 4 for PCP) was 2.7. In addition, the BCFs were proportionate to the estimated log P values [7]. Because replicate tests were not conducted within each laboratory, no estimate of variance could be determined. The mean H/L ratio of 2.7, however, indicates good reproducibility. Lipid content of oysters can vary considerably, and for lipophilic chemicals this may cause bioconcentration

factors to vary proportionately. Lipid content of oysters in these tests, where available, varied little.

Uptake rates for PCP and TCB, as calculated by NONLIN [*9*], varied by factors of 6.2 and 5.8, respectively. The time calculated for 90% of steady state for p,p′-DDE exceeded the exposure period (28 days) for three of the four participating laboratories. This was expected because of the high log P value for the chemical (5.69). As with the BCF values, uptake rate correlated with log P values (for example, the lower the log P, the shorter the time to steady state).

Depuration rates for each chemical generally varied among laboratories more than the BCF, but less than uptake rates. The H/L ratios of the time (in hours) required for 90% depuration of PCP, TCB, and p,p′-DDE were 3.1, 5.3, and 1.6, respectively. Oysters from Laboratory 4 showed consistently lower depuration rates for all three chemicals.

The bioconcentration test method can be improved by providing specific methods for lipid analyses. Work and Work [*10*] provide a general reference; however neutral lipids may be obtained using the extraction methods of Schimmel et al [*11*], except that the combined extract should not be cleaned with florisil. Polar and total lipids may be obtained using the homogenization procedures of Schimmel et al [*11*], but a mixture of chloroform/methanol/water should be used as suggested by Bligh and Dyer [*12*].

In summary, Draft 9 of the ASTM bioconcentration test method provided reproducible results, particularly with the steady state BCF values. This occurred despite the differences observed in how strictly each laboratory adhered to the method, wide variety in testing experience, the wide geographical distribution, and the resultant differences in dilution water characteristics of the participating laboratories.

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